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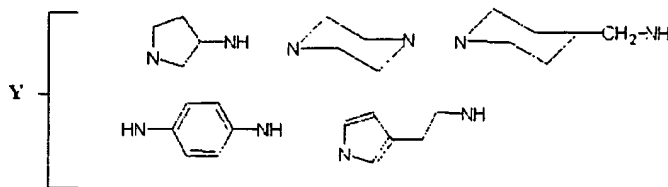
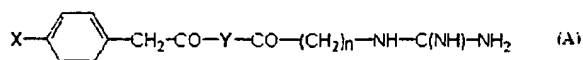
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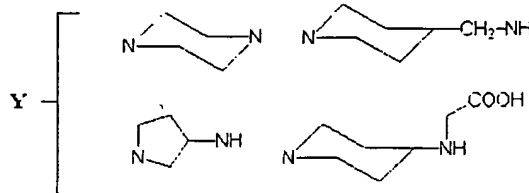
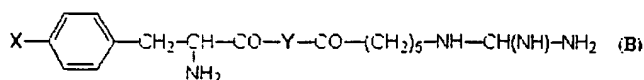
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(54) Title: NON PEPTIDE MIMETICS BASED ON THE ACTIVE SEQUENCE S₄₂FLLR₄₆ OF THE THROMBIN RECEPTOR
FOR THE TREATMENT OF THROMBOSIS AND CANCER

X = H, F

n = 1-5

(57) Abstract: The invention relates to novel non peptide compounds (mimetics) based on a thrombin receptor sequence and novel methods for the synthesis of these compounds. These compounds act as agonists or antagonists in a variety of cells including endothelial cells, blood platelets, vascular smooth muscle cells and tumor cells. They are useful in the treatment of thrombosis and cardiovascular diseases and modulation of angiogenesis for cancer treatment or wound healing.



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TITLE

NON PEPTIDE MIMETICS BASED ON THE ACTIVE SEQUENCE S₄₂FLLR₄₆
OF THE THROMBIN RECEPTOR FOR THE TREATMENT OF
5 THROMBOSIS AND CANCER

TECHNICAL FIELD OF THE INVENTION

The invention relates to novel non peptide compounds based on a thrombin
10 receptor sequence and novel methods for the synthesis of these compounds. These
compounds act as agonists or antagonists in a variety of cells including endothelial
cells, blood platelets, vascular smooth muscle cells and tumor cells. They are useful in
the treatment of thrombosis and cardiovascular diseases and modulation of
angiogenesis for cancer treatment or wound healing.

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INTRODUCTION-BACKGROUND OF THE INVENTION

Thrombin is a multi-functional protein. It is the key enzyme involved in
haemostasis, and plays important roles in many cellular and physiological events.
20 First, in the coagulation cascade, thrombin converts fibrinogen into fibrin, which is an
integral part of most clots. In addition, thrombin is known to act directly on cells in the
blood and on the interior blood vessel wall and specifically to activate platelets to form
clots. Thrombin-induced platelet aggregation is particular important for arterial
thrombus formation, a process that causes myocardial infarction and some forms of
25 unstable angina and stroke. Furthermore, thrombin promotes inflammation and other
cellular activities. Thrombin can elicit mitogenic responses to vascular smooth-muscle
cells. Additionally, thrombin is able to elicit responses from to cell types as diverse as
macrophages, monocytes and neutrophils. Finally, thrombin is a potent angiogenic
factor thus regulating tumor progression and metastasis.

30 Most of these biological activities of thrombin are mediated through its specific
functional G-protein-linked cell surface receptors, which have been cloned from human
platelets and endothelial cells, rat vascular smooth muscle cells, and hamster lung
fibroblasts¹⁻³. The activation mechanism of target cells by thrombin involves a

proteolytic cleavage of the extracellular N-terminal bond between Arg₄₁ and Ser₄₂ of the thrombin receptor. The newly generated N-terminus unmasked by thrombin-induced cleavage serves as a tethered ligand⁴ which binds intramolecularly to effect receptor activation^{5,6}. In support of this model, synthetic peptides (Thrombin Receptor
5 Activating Peptides, TRAP) corresponding to at least the five residues of the new N-terminus (S₄₂FLLR₄₅) are effective in mimicking many of the actions of thrombin⁷⁻¹⁰. Structure-activity relationships (SAR) and alanine scan experiments have indicated that Phe₄₃ and Arg₄₅ are the most important amino acids of the receptor-derived peptide SFLLR for its activity in smooth muscle^{11,12} and for its ability to aggregate platelets^{7,8}.
10 Recent conformational studies have shown that a cyclic conformation for SFLLR in which the Phe and Arg residues cluster together to form a primary pharmacophore motif may be required for the biological activity of the peptide¹². The above proposed cyclic model was validated through design and synthesis of cyclic derivatives of SFLLR which were found to be biologically active, confirming the importance of
15 phenyl, guanidino and amino groups as pharmacophores^{13,14}.

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STATE OF ART OF THE INVENTION

This invention concerns novel non-peptide compounds based on a thrombin receptor sequence and methods of synthesizing these novel compounds. We envision
5 that these non-peptide mimetics can be used orally as therapeutics in inhibiting angiogenesis for cancer treatment. This can be accomplished with concentrations of these compounds ($\sim 90 \mu\text{M}$), at which platelet aggregation and the blood coagulation cascade are not affected.

10 1. Conformational Model

A conformational model for SFLLR, the shortest sequence to mimic thrombin, is described in which the side chains of residues Phe and Arg together with the N-terminal NH_2 group form a cluster. These side chains together with their conformational distances derived from NOE studies provide for the pharmacophoric
15 groups of Thrombin Receptor Activating Peptides (TRAP) mimetics.

2. Rational Design

Many peptides are often intrinsically limited as drugs since they usually exhibit poor solubility, stability and/or bioavailability. Efforts to improve the metabolic
20 stability, duration of action and bioavailability of such peptides has stimulated the search for new peptidomimetic drugs. Peptide mimetics represent peptide-like molecules, which can mimic the binding of natural peptides at their native receptor or enzyme targets. In the present invention the structural requirements of the native pentapeptide SFLLR (P5) have been incorporated onto different molecular scaffolds in
25 a particular spatial order that is in agreement with the previously defined bioactive topology. The described compounds have been designed rationally¹⁵ in order to mimic the active cyclic conformation adopted by P5. In this conformation the Phe and Arg residues are in a close proximity on the same side of the cyclic ring. Replacing the aliphatic residues of the active peptide core with a molecular template would be one
30 way of stabilizing and testing compounds able to adopt the above cyclic conformation. Furthermore, the existence of a primary NH_2 group has been proved critical for receptor activation by TRAPs

3. Novel Structures

One series of compounds have been designed using the template compounds "A" and "B" as shown in scheme 1 and scheme 2

5 DETAILED DESCRIPTION OF THE INVENTION

1. Synthesis of Thrombin Receptor Activating Peptide (TRAP) non-peptide mimetics

10 "Group A" mimetics

The synthesis of the representative analogue THO7 of group A TRAP mimetics was accomplished as it is shown in Scheme 3. Briefly, 3-(phenylacetyl-amido)pyrrolidine was synthesized by coupling 3-aminopyrrolidine and phenylacetic acid with DCC and HOBt as coupling reagents. That product was the
15 only one obtained, as found from HPLC, ¹H NMR and ¹³C NMR experiments. Specifically, the primary amine of 3-aminopyrrolidine found as the most active in the above reaction. That was confirmed by ¹³C NMR spectrum having as references the different C-absorbances of amidic bonds: CO=NH (δ =169.5 ppm), CO=N (δ =168.1 ppm). Boc- ϵ -Aminohexanoic acid was then incorporated in the NH of pyrrolidine
20 aided by the use of DCC and HOBt under basic conditions (DIEA). Boc-deprotection was accomplished with trifluoroacetic acid giving the free amine salt. Guanylation of the primary amine using 1*H*-pyrazole-1-carboxamide hydrochloride afforded compound THO-7, which was purified by recrystallization (MeOH/Acetone/Et₂O). The synthesis of compounds of the DOL, PET, MAR and MIC series was carried out
25 by an analogous procedure.

"Group B" mimetics

The synthesis of the group B thrombin receptor derived peptide analogues (Scheme 4) was accomplished using solid phase methods and the 2-chlotrityl chloride
30 resin as solid support. Solid phase organic synthesis offers the advantage of simple and fast work up procedures as compared to the classical solution chemistry. Attachment of the amino group of phenylalanine (L-Phe) to the resin (2.27 mequiv. Cl⁻/g of resin) was achieved by refluxing using TEA in CH₂Cl₂ solution and after protection of the

carboxyl group of Phe with $(\text{CH}_3)_3\text{SiCl}$. The loading of Phe to the resin was certified by the Kaiser ninhydrin test. The Phe attached to the resin was first activated with DIC and HOBt and then divided among three reaction vessels where it was coupled with three different diamines (e.g. piperazine, 4-aminomethyl-piperidine and 3-aminopyrrolidine) in DMF and in the presence of DIEA. Couplings with Fmoc- ϵ -Ahx-OH (2.5 equiv.) were aided by the use of DIC and HOBt under basic conditions (DIEA) using minimum of DMF. Fmoc group removals were carried out by treatment with 20% piperidine/DMF for 30 min. The guanidino group was incorporated using 1*H*-pyrazole-1-carboxamide hydrochloride in DMF/DIEA in the last solid phase chemistry step before cleavage. The resin was cleaved with 10% TFA/ CH_2Cl_2 for 15 min at room temperature. The crude products were purified by HPLC.

2. Biological Activities

15 Modulation of Angiogenesis

We have evaluated the effects of DOL1 and DOL5 in the CAM system of angiogenesis. The two compounds at 90nmol/disc inhibited angiogenesis as evidenced by morphological evaluation and the extent of collagenous protein biosynthesis under the disc containing the test compound as compared to controls. When used in combination, 90 nmol of these compounds, with thrombin (1.0 IU/disc), the angiogenic effect of thrombin was completely abolished.

In addition the synthetic antagonists to the activated thrombin receptor of DOL series (DOL1, DOL2, DOL3, and DOL5) were evaluated for their effects on endothelial cell progelatinase A activation (Figure 1). This metalloproteinase is secreted in the growth medium of human umbilical cord endothelial cells (HUVECS) in culture and appears at 72 KDa collagenolytic zone in zymograms. A second zone appears at 62 KDa corresponding to the activated form of progelatinase A. Under control conditions the activated gelatinase (62 KDa) was about 8% of the 72 KDa zone. When the HUVECS are treated with thrombin (1.0 IU/ml), for 24 hours, the 62 KDa zone increased to an extent of approximately 50% of the 72 KDa zone.

When DOL1, DOL2, DOL3, and DOL5 were present at concentrations of 90 μM in the culture medium of HUVECs the zone of 68 KDa of the activated gelatinase is greatly reduced. Furthermore, when used in combination with either thrombin (1.0

IU/ml) or SFLLR (500 μ M) at 90 μ M each, they all antagonized the activating effect of thrombin and SFLLR. It is of interest that this effect is evident at concentrations (90 μ M), which have no effects on platelet aggregation.

5 Rat Aorta Relaxation

TRAP mimetics from group B were tested for biological activity in isolated aortic rings with intact endothelium pre-contracted with phenylephrine and were found capable to produce a dose-dependent relaxation at concentrations as low as 1-10 μ M. In this system, nitric oxide synthase blocker L-NAME, inhibited TRAP induced aortic
10 relaxation. The compound NAT5 completely blocked the relaxation effect after pre-treatment with L-NAME. This indicates that all relaxing properties of NAT5 are mediated by nitric oxide (NO) release. In contrast, MEX5 retained both NO-dependent and NO-independent relaxing properties, whereas NEC5 had NO-dependent relaxing properties twice as potent as NO-independent ones.

15 In preparations with denuded endothelium, the TRAP mimetics also had relaxing effect of variable magnitude. Thus, NAT5 had practically no relaxing effect, indicating in a complementary manner, that all the relaxing properties of this substance were mediated by the endothelium. MEX5's endothelium-independent effect was 31%, whereas NEC5's, a value of 14.5% was found for the endothelium independent relaxing
20 effect (Figure 2).

Generally, the magnitude of the relaxation induced by a given TRAP mimetic was higher in preparations with endothelium than in de-endothelized preparations. The two types of relaxation were very close or equal in the preparations pre-treated with L-NAME.

25 From group B, NAT5 was a significantly stronger stimulator of nitric oxide release (40% relaxation at 0.1 mM), than the other two compounds. No significant differences were observed within MEX5 and NEC5 regarding their relaxing activities (27.5% and 24.8% respectively, at 0.1 mM). The ED_{50} of NAT5 (3.65 ± 0.8 μ M) was one order of magnitude lower than the ED_{50} of NEC5 (26 ± 1.2 μ M) and of MEX5
30 (13.8 ± 4.1 μ M). This indicates that NAT5 has a higher affinity for the endothelial thrombin receptor than MEX5 and NEC5, which have the same magnitude affinity.

Platelet Aggregation

The effects of thrombin on platelets are mediated by thrombin receptors. Since in our experience and that of other investigators¹⁶ rat platelets are poorly reactive to thrombin and to SFLLR-NH₂, we chose human platelets to evaluate the ability of three representative compounds from group A (DOL5, PET7 and THO7) to induce platelet aggregation or inhibit the thrombin- or SFLLR-NH₂-induced platelet aggregation¹⁷. All these compounds inhibited thrombin induced platelet activation when thrombin was used at low concentration (up to 0.2 NIU/mL or 2 nM) which requires thrombin's anion-binding exosite¹⁸ and occurs via activation of the thrombin tethered-ligand receptor¹⁶. However at higher concentrations of thrombin, (up to 0.3 NIU/mL or 3 nM) when platelet activation was achieved throughout an independent of thrombin's exosite and mediated by a moderate affinity (KD=10nM) binding site¹⁸, thrombin could overcome such inhibition for both PET7 and DOL5 but not for THO7 (Figure 3).

Platelet activation with low concentrations of thrombin was almost totally prevented with three TRAP mimetics of the group B (MEX5, NEC5 and NAT5), at a relatively high concentration (0.5 mM - 1 mM) for both NEC5 and NAT5 but not for MEX5. MEX5 inhibited permanently thrombin-induced platelet activation at a 4-fold smaller concentration (0.125 mM) than the other two TRAP mimetics. At higher concentrations (up to 0.3 NIU/mL), thrombin could partly overcome all three TRAPs inhibitory activities when TRAP mimetics were used at low concentrations. By contrast, higher concentrations of TRAPs (up to 1 mM for NAT5 and NEC5 and 0.5 mM for MEX5) completely inhibited thrombin-induced platelet activation even when thrombin was used up to 3 nM (Figure 4). Therefore, of these new TRAP mimetics NEC5 and NAT5 have limited efficacy in blocking a thrombin-stimulated platelet activation when used up to 0.25 mM even though NEC5 up to 0.25 mM enhanced weakly platelet activation. MEX5 at a concentration 0.25-0.5 mM caused a complete and permanent blockage of thrombin-induced platelet activation.

Taking into consideration that (a) the effects of thrombin on platelets are mediated through its specific receptor, (b) SFLLR-NH₂ directly activates the thrombin receptor and (c) the compounds had no effect on collagen-induced platelet aggregation, we may come to the conclusion that the compounds tested with the platelet aggregation studies exert their biological activity through specific interactions with the thrombin receptor rather than by inhibiting thrombin's enzymatic activity or stimulating other

type of receptor(s)¹⁹.

Several tentative reasons to explain the difference between the thrombin-like agonistic effect of these compounds in the rat aorta relaxation assay and the anti-thrombin effect in the plasma aggregation assay can be proposed here. Firstly, different types of thrombin receptor may exist in different species. In this regard, human but not rat and dog platelets are activated by SFLLR peptides¹⁶. Secondly, different types of thrombin receptor(s) and different activating pathways may be stimulated in platelets and in endothelial cells. Indeed, similar results to our findings have been reported for Mpa-peptides²⁰. Nevertheless, the existence of a molecule that could both inhibit platelet aggregation and augment vasorelaxation would be of paramount clinical significance and it could potentially be used to prevent vascular re-occlusion after thrombolytic therapy of acute myocardial infarction or after coronary artery angioplasty²¹.

3. Utility

Thrombin active site inhibitors prevent most of thrombin's actions and thrombin inhibitors, such as argatroban, hirulog and hirudin have been found useful in preventing thrombosis in a number of animal models²² and have therapeutic utility in a variety of clinical situations that are currently under study²³. However, thrombin carries out of a wide variety of processes activating a number of procoagulant (activation of clotting fibrinogen, factors V, VIII, IX, and XIII) and anticoagulant (activation of protein C) actions in addition to the thrombin receptor-mediated cellular actions²⁴. A receptor selective agent might allow the clotting actions of thrombin. Such agents, should they be potent and selective, might have interesting efficacy by virtue of their ability to spare the inhibition of protein C activation. As a result, a receptor antagonist might be efficacious and potentially safer with regard to bleeding complications, since not all of the thrombin's actions would be inhibited.

Similarly, modulation of the angiogenic action of thrombin for therapeutic purposes with the currently available anti-coagulants is not practical. Several limitations in their actions preclude the use of the currently available anti-thrombotic drugs for clinical application for suppression of angiogenesis in cancer and other angiogenic diseases for which long-term treatment is required. In the same way, the

use of thrombin as an angiogenic factor in wound healing and ischaemic conditions is not possible because of its thrombogenic effect. Therefore, other agents that mimic or antagonize the angiogenic action of thrombin, which are not thrombogenic or interfere with blood coagulation are desirable. In view of the current interest in therapeutic
5 modulation of angiogenesis and the role of thrombin as an angiogenic factor, the need for developing thrombin receptor mimetics and thrombin receptor antagonists for this action of thrombin in cells is obvious.

The compounds of the present invention are suitable for in vivo administration. They are soluble and their small size may increase their bioavailability make them
10 suitable for oral administration. The formulation of suitable compositions for oral administration can be undertaken by those skilled in the art.

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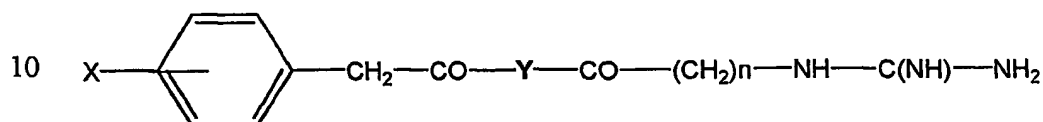
CLAIMS

1. Conformational Model

A conformational model for SFLLR in which the side chains of residues Phe and Arg together with the N-terminal NH₂ group form a cluster.

2. Novel Non Peptide TRAP Mimetics with Two Pharmacophoric Groups

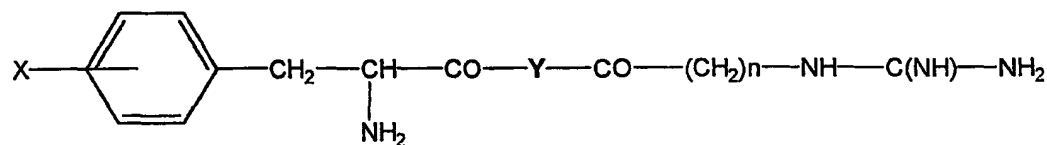
Novel compounds as shown below



where X = H, F; Y = piperazine, 4-aminomethylpiperazine, 3-aminopyrrolidine, 1,4-diaminobenzene, histamine and imidazole; n = 1, 2, 3, 5.

3. Novel Non Peptide TRAP Mimetics with Three Pharmacophoric Groups

Novel compounds as shown below

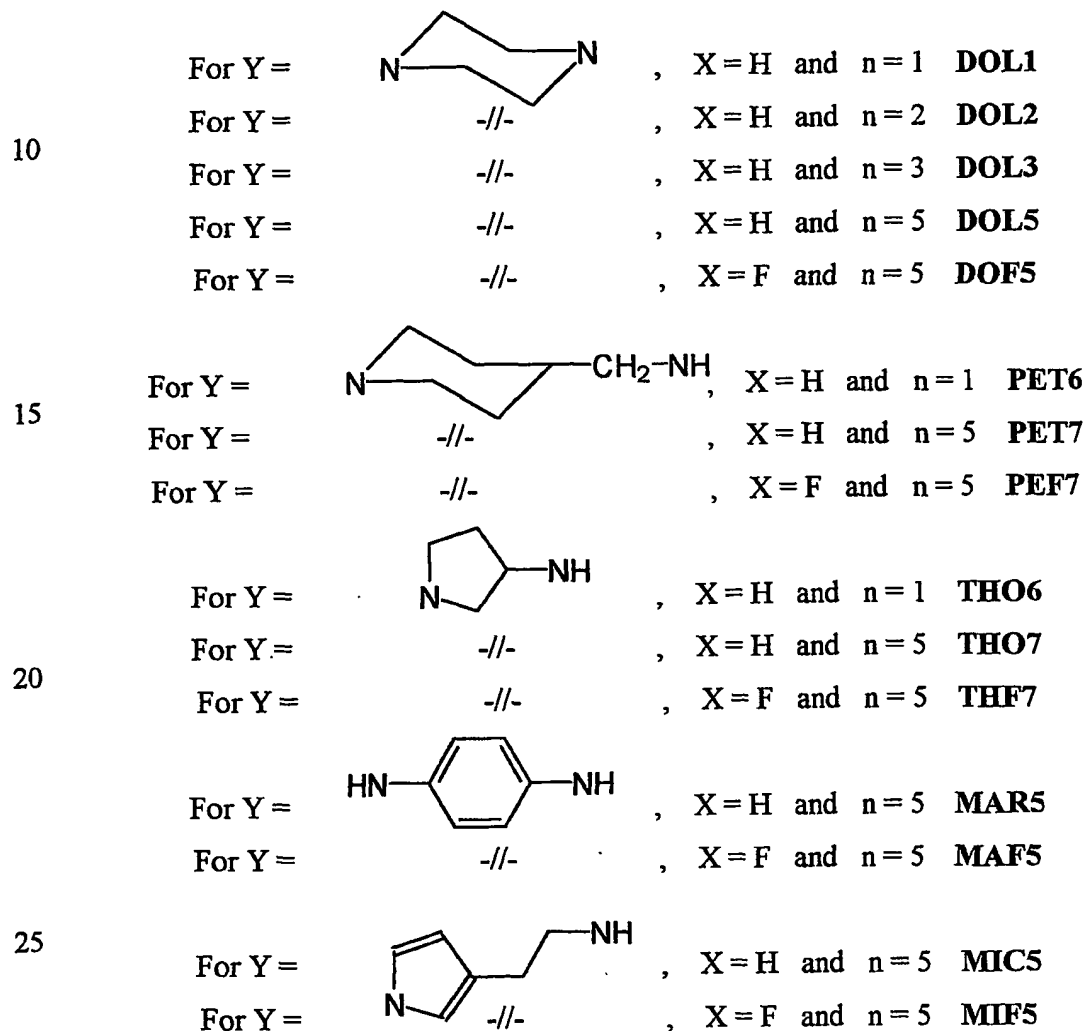
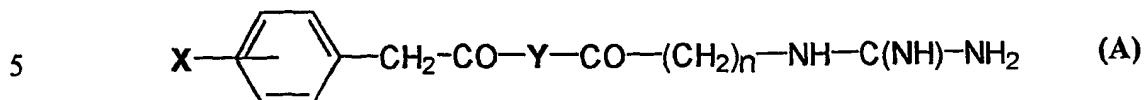


20 where X = H, F; Y = piperazine, 4-aminomethylpiperazine, 3-aminopyrrolidine, pyridineglycine and imidazole; n = 1, 2, 3, 5.

4. Biological Evaluation of TRAP mimetics

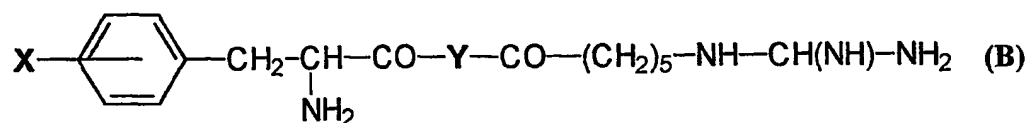
The above synthesized compounds were found to act as antagonists on angiogenesis in the chick chorioallantoic membrane (CAM) system and also as inhibitors in the activation of progelatinase A (MMP-2) in the culture medium of human umbilical cord endothelial cells (HUVECs). They were also found active in the rat aorta relaxation assay and the platelet aggregation study. The above compounds could be used orally for the treatment of cancer, thrombosis and cardiovascular diseases.

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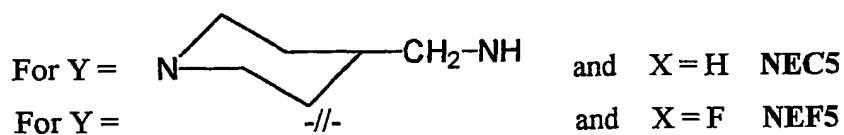
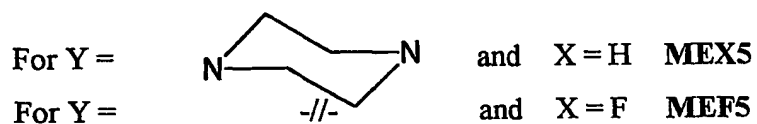


Scheme 1

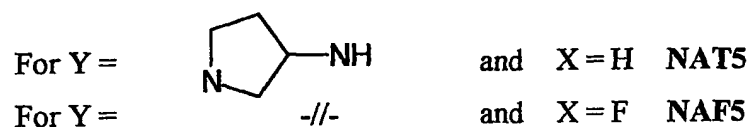
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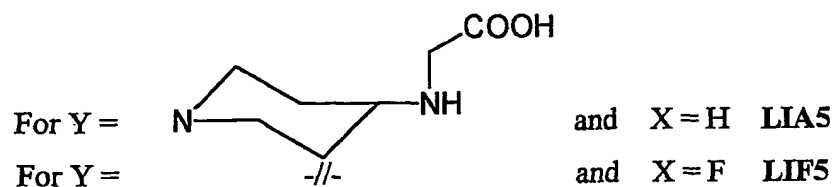
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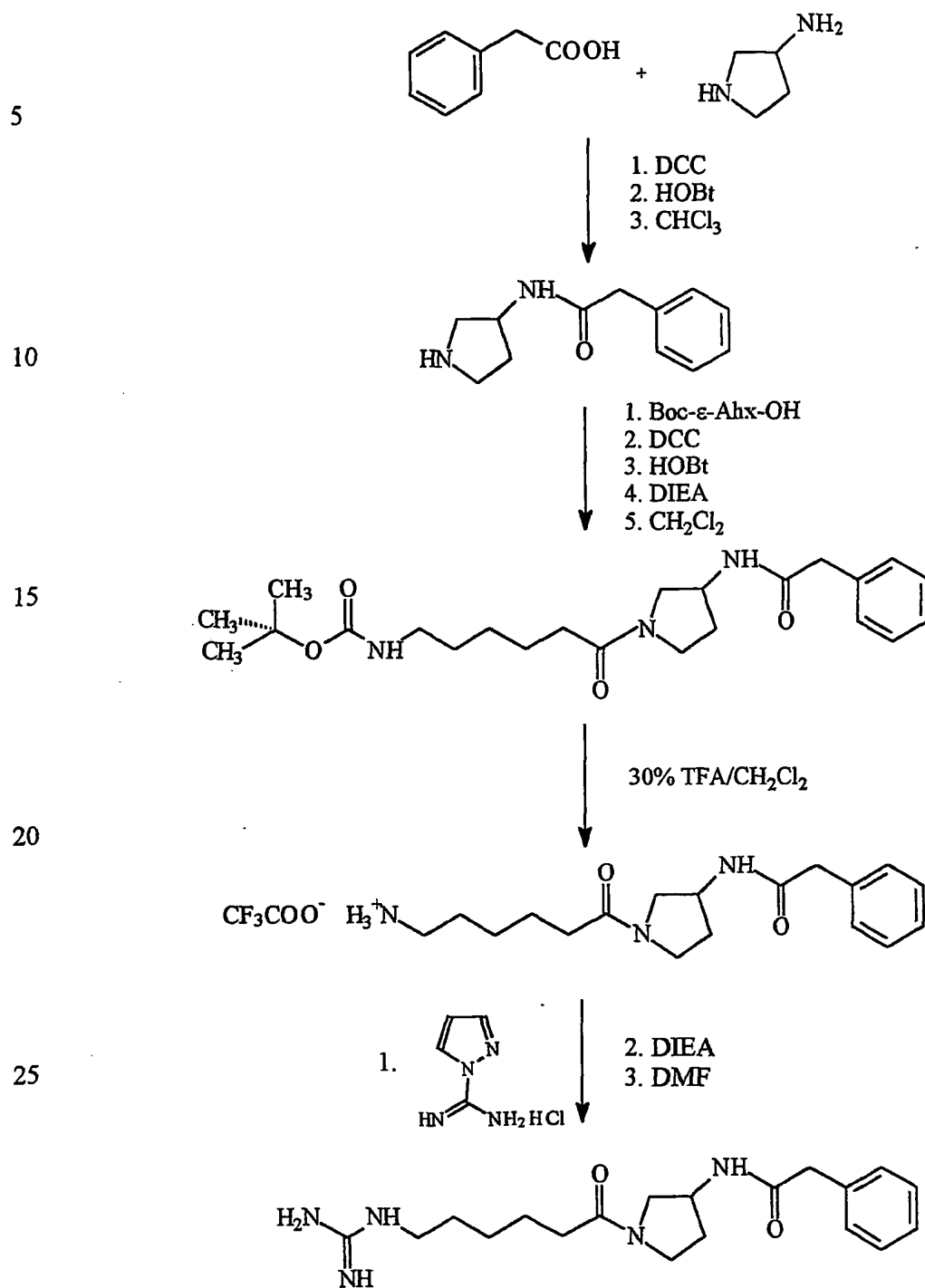
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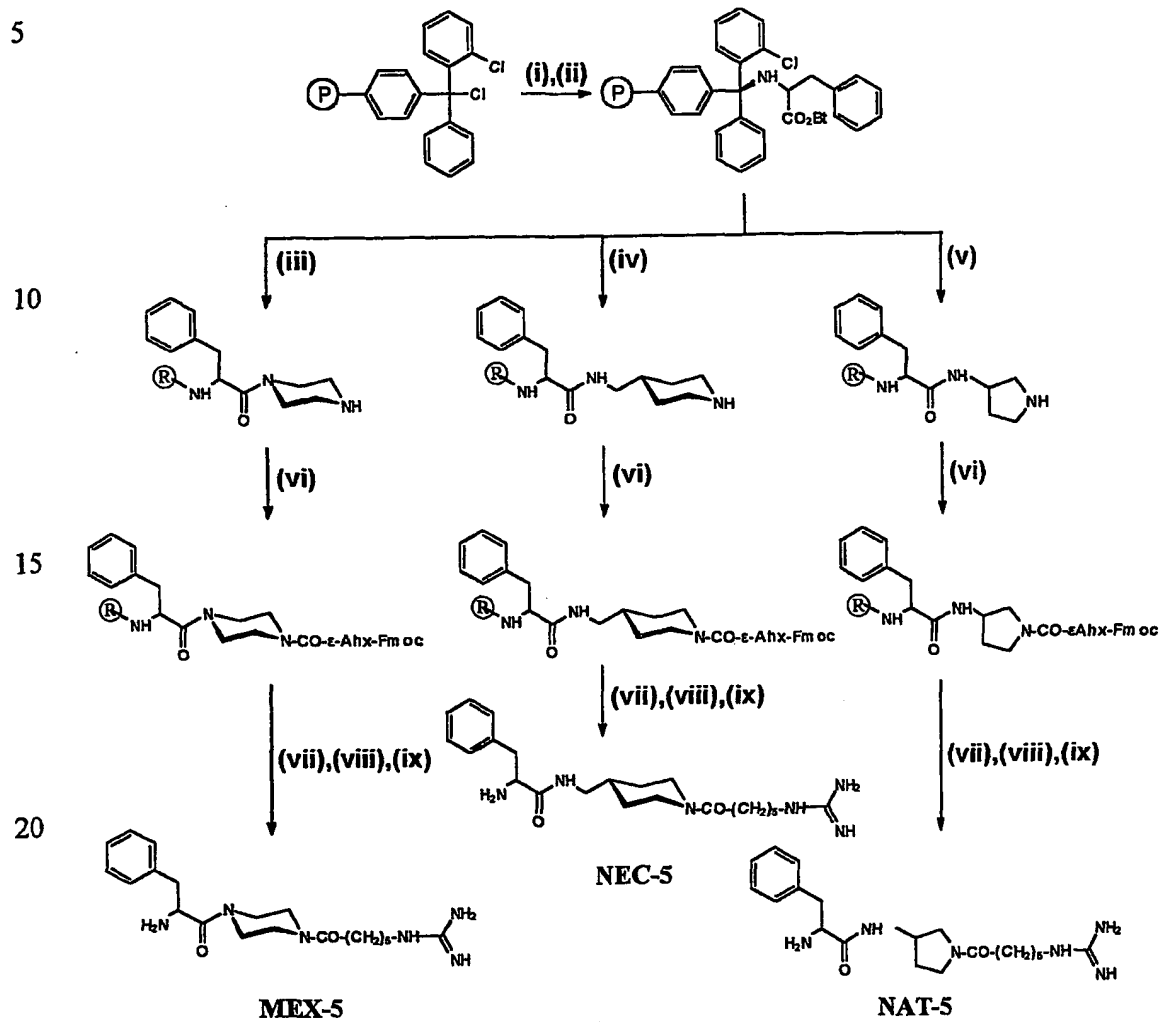
Scheme 2

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Scheme 3



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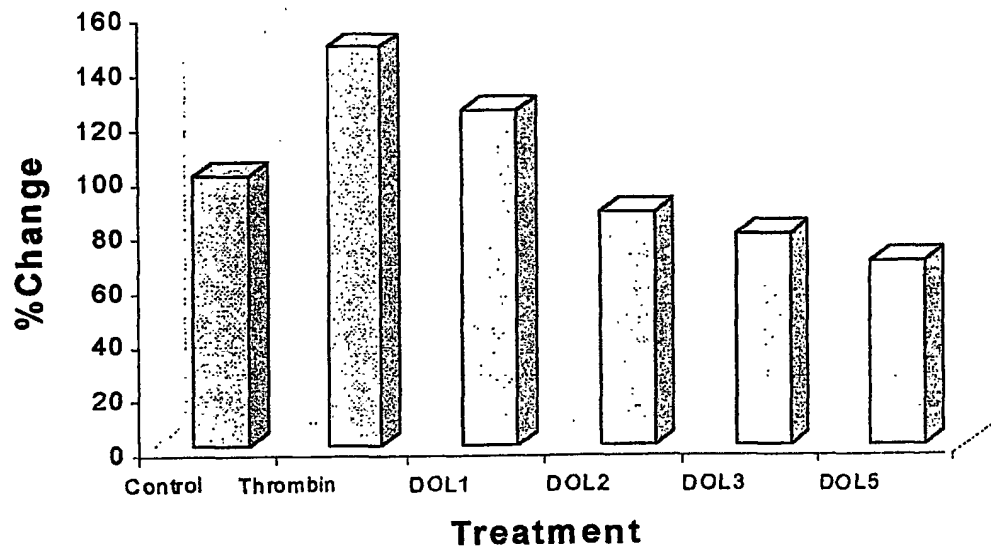
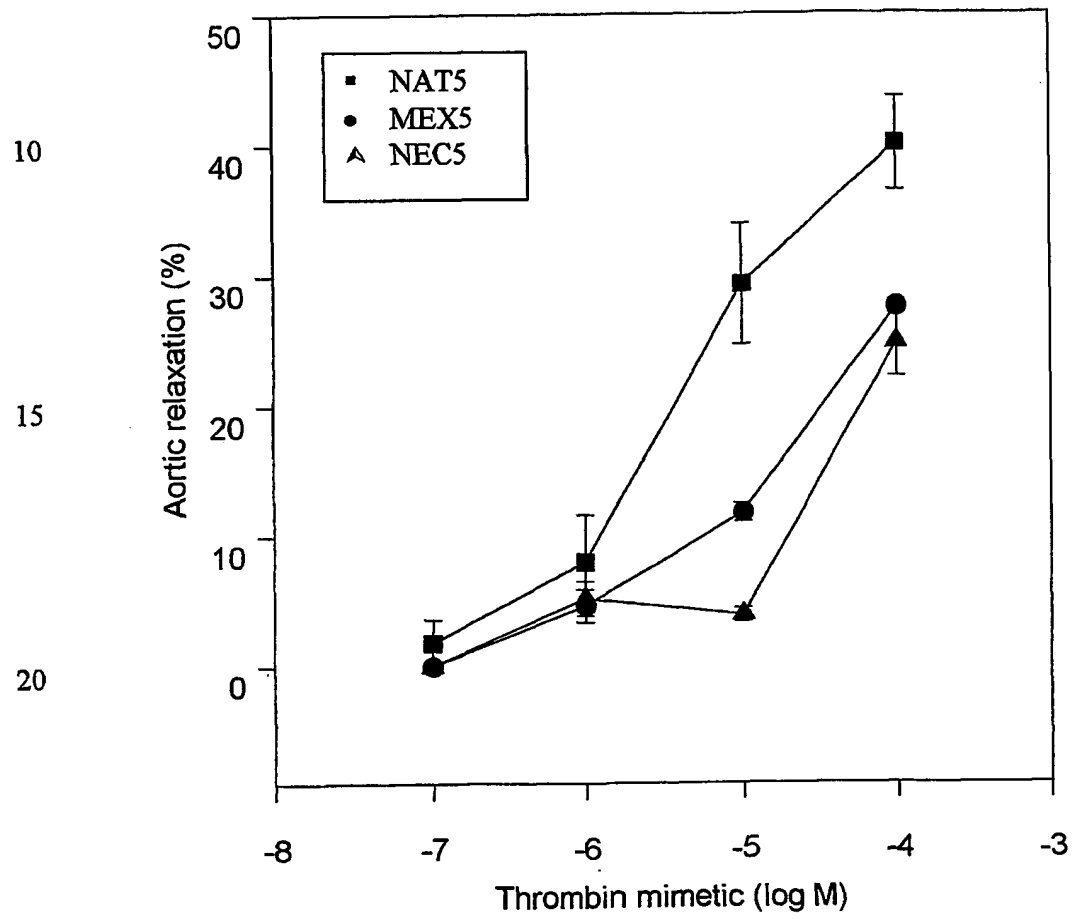


Figure 1

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Figure 2

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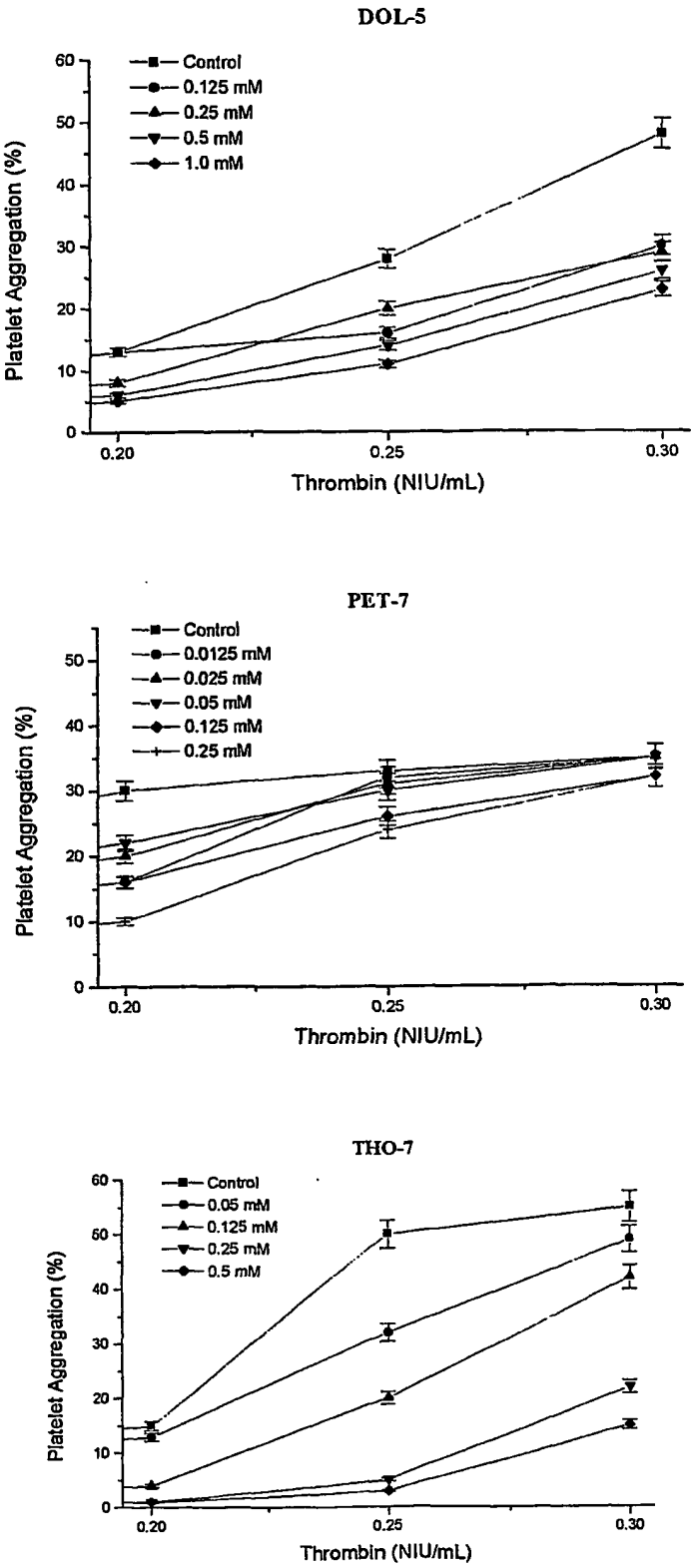


Figure 3

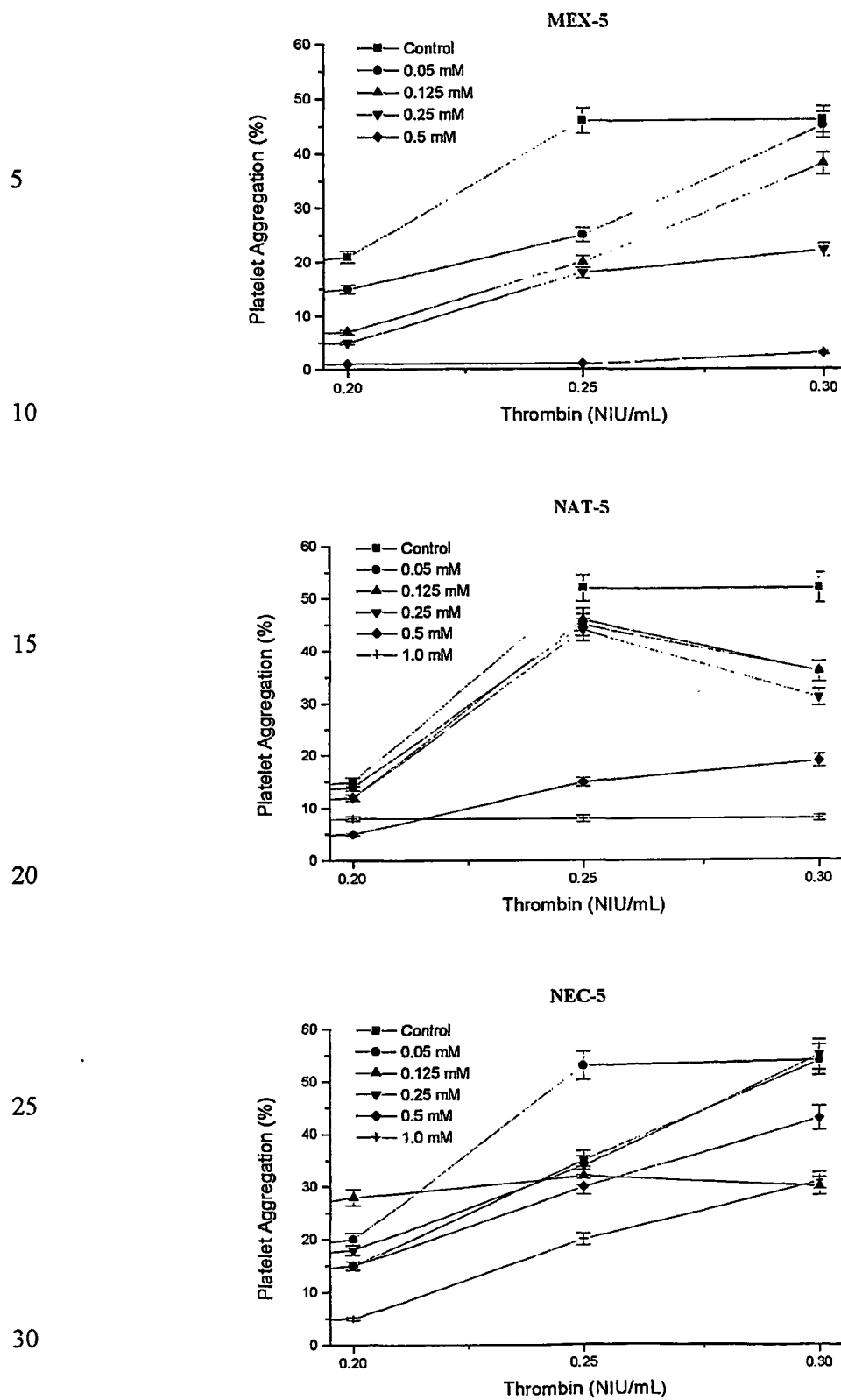


Figure 4

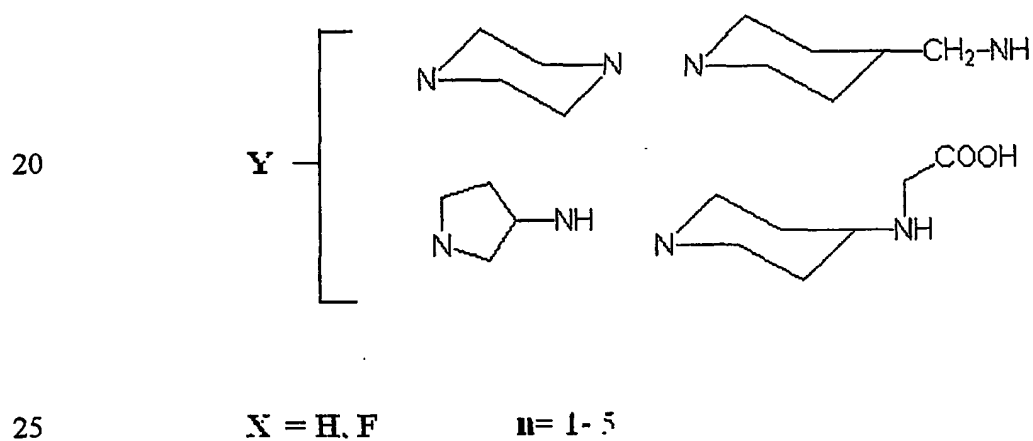
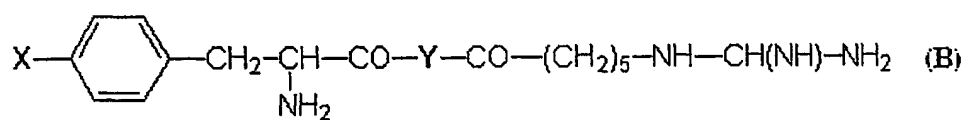
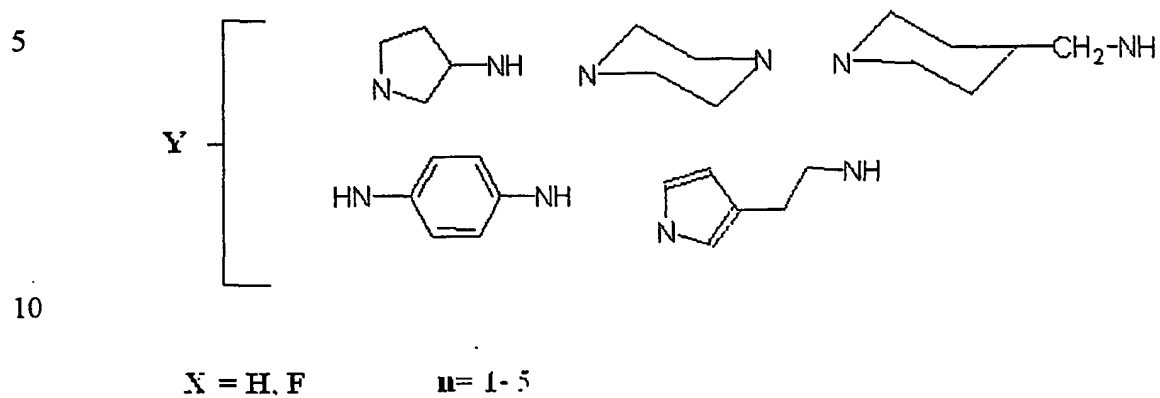
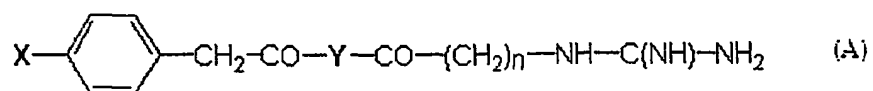


Figure 5

INTERNATIONAL SEARCH REPORT

 Int. Application No
 PCT/GR 01/00015

A. CLASSIFICATION OF SUBJECT MATTER

 IPC 7 C07D295/185 A61K31/40 A61P35/00 C07C279/14 C07D207/14
 C07K7/06 C07D211/18 C07D207/335

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07D C07C A61K A61P C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALEXOPOULOS, KOSTAS ET AL: "Synthesis and biological activities of thrombin receptor derived non peptide mimetics: importance of phenyl/guanidino proximity for activity" PEPT. 1998, PROC. EUR. PEPT. SYMP., 25TH (1999), MEETING DATE 1998. EDITOR(S): BAJUSZ, SANDOR;HUDECZ, FERENC. PUBLISHER: AKADEMAI KIADO, BUDAPEST, HUNG. , pages 650-651, XP001015506 the whole document	1,2,4
A	---	3
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☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
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- * & * document member of the same patent family

Date of the actual completion of the international search

21 September 2001

Date of mailing of the international search report

08/10/2001

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Authorized officer

Sánchez García, J.M.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GR 01/00015

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALEXOPOULOS, KOSTAS ET AL: "Design and synthesis of thrombin receptor-derived nonpeptide mimetics utilizing a piperazine scaffold" BIOORG. MED. CHEM. (1999), 7(6), 1033-1041 , XP001010695 page 1033 -page 1034	1,2,4
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X	MISSIRLIS, ELEFThERIA ET AL: "Peptidomimetic thrombin receptor analogs: effects on angiogenesis and metalloproteinase activation in endothelial cells" BIOMED. HEALTH RES. (1999), 22(BIOACTIVE PEPTIDES IN DRUG DISCOVERY AND DESIGN: MEDICAL ASPECTS), 71-79 , XP001015504 page 72 -page 73	1,2,4
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X	ALEXOPOULOS, KOSTAS ET AL: "A comparative SAR study of thrombin receptor derived non peptide mimetics. Importance of phenyl/guanidino proximity for activity" AMINO ACIDS (1998), 15(3), 211-220 , XP001010683 page 212 -page 213	1,2,4
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X	DATABASE CA 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; MATSOUKAS, JOHN ET AL: "Conformational analysis of the thrombin receptor agonist peptides SFLLR and SFLLR-NH2 by NMR: evidence for a cyclic bioactive conformation" retrieved from STN Database accession no. 126:340655 XP002177980 abstract & J. PROTEIN CHEM. (1997), 16(2), 113-131 , ---	1

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INTERNATIONAL SEARCH REPORT

Int. Patent Application No.

PCT/GR 01/00015

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>ALEXOPOULOS, KOSTAS ET AL: "Design, Synthesis, and Modeling of Novel Cyclic Thrombin Receptor-Derived Peptide Analogues of the Ser42-Phe-Leu-Leu-Arg46 Motif Sequence with Fixed Conformations of Pharmacophoric Groups: Importance of a Phe/Arg/NH2 Cluster for Receptor Activation and Implications in the Design of Nonpeptide Thromb"</p> <p>J. MED. CHEM. (2001), 44(3), 328-339 , XP002177978</p> <p>page 328 -page 329</p> <p>-----</p>	1-4